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Master: **A Study of the Effects of Nicotine on Gestation in the Rat with Paricular Reference to Implantation and the Time of Onset of Parturition.**

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1. Present State of Knowledge

The results of numerous studies have established that nicotine may interfere with the normal course of pregnancy in the rat. It has been claimed that the administration of nicotine to pregnant rats results not only in reduced litter size (20-22, 38, 40, 42) and litter weight (2-5, 15, 18, 19, 25, 26) but also in increased frequency of stillbirths (4, 11, 22) and infant mortality (2-4, 10, 11, 14, 20, 32, 34).

Among the most systematic and informative studies of the actions of nicotine on pregnancy are those of Becker et al., (2-5). When twice daily sc injections of 3.0 or 5.0 mg/kg of nicotine were administered throughout pregnancy, the onset of parturition was delayed as much as 3 to 5 days, the mean delay resulting from the higher dose being 2 days (4). Also, the young weighed less and were retarded in development relative to controls.

In another study, Becker et al , (5) again employed the 5 mg/kg dose to prolong gestation. However, for comparison non-nicotine treated rats were given chorionic gonadotrophin in order to prolong pregnancy. Although equivalent delays in parturition were achieved in the two groups (all were delivered by Ceasarean section of Day 21, 22, 23, or 24), the characteristic of the young were markedly different. Young born following hormonally prolonged gestation evidenced the typical post-maturity syndrome, i.e., greater body weight and development compared with

neonates born at term. In contrast the neonates of nicotine treated rats, though subjected to an equivalent delay in parturition, were markedly lighter in weight and retarded in development. [illegible] More recently, Hudson and Timiras (17) investigated the effects of nicotine on reproductive capacity and length of gestation, as well as on litter size and viability. Injection of 5 mg/kg of nicotine, administered sc twice daily throughout pregnancy, prolonged gestation by 2-3 days; the most protracted pregnancy was of 27 days duration. However, in contrast with the results of Becker et al., (4, 5) birth weights were not statistically different between treated and control rats. Furthermore, no mention was made of retarded development.

Birth weights of nicotine treated rats did not differ from those of control rats in spite of prolonged gestation, which suggests that either the rate of fetal growth was reduced throughout gestation, or that the rate of growth was normal but slow in starting. The failure or prolonged gestation in nicotine treated rats to result in larger and more mature neonates favors the latter interpretation and suggests that nicotine delays implantation. A delay in uterine sensitivity would produce a delay in implantation which in turn could be reflected as a delay in parturition. Such a delay in nidation would be consistent with the birth of young of normal weight and development as reported by Hudson and Timiras (17).

However, other explanations for the observed delay in parturition are possible. The prolongation in gestation reported by Becker et al. (4, 5) was associated with reduced fetal bulk. A reduction in fetal mass may contribute toward a delay in parturition (29). It is also possible that nicotine reduces fetal mass by reducing the magnitude of uterine sensitivity to the blastocyst, thereby diminishing litter size. Reduction in litter size following administration of nicotine has been observed (19, 33, 35). Another possibility is that nicotine may delay the onset of parturition, not by delaying implantation but by retarding the rate of fetal growth by suppressing maternal food intake (4). The proposed experiments are designed to discriminate between these various possibilities.

2. Specific Objectives

The primary objective of the experiments embodied in this proposal is to gain a greater understanding of the means by which nicotine exerts disruptive effects on pregnancy in the rat, and more particularly, how the alkaloid brings about reduction in litter size and delay in parturition. The following experimentally testable hypotheses are proposed:

I. That nicotine reduces uterine sensitivity to implantation of the blastocysts.

II. That nicotine delays the normal time of onset of uterine sensitivity to implantation of the blastocysts.

III. How That the nicotine induced delay in time of parturition in rats is a reflection of the delay in the time of implantation of the blastocyst. reported was associated with reduced fet

The following is a brief resume of why it is reasonable to assume that nicotine may alter endometrial physiology:

nic Ample evidence has been marshalled to support the long held contention that uterine sensitivity to decidualization is a reliable model for studying uterine receptivity to the rat blastocyst (13). Because induction of the decidual cell reaction has proved so useful in investigating the process of nidation itself, considerable work has been directed toward determining the physiological and metabolic conditions which are necessary to support the response.

va Not only has the temporal course of uterine sensitivity to decidualization been worked out in detail (38), but the hormonal balance requisite for optimal deciduoma formation has been established (37). It is now known that the magnitude of the decidual response as well as the timing of sensitivity is regulated by an estrogen-progesterone interplay. Although a decidual response may be evoked in the presence of progesterone alone, it is submaximal in magnitude; estrogen must also be present for maximal responsivity to occur. Although estrogen potentiates the uterine sensitizing effect of progesterone, it also curtails the period during which a maximal decidual reaction can be evoked (38).

Therefore, whereas progestogens render the endometrium receptive to the blastocyst, estrogen regulates the magnitude and duration of receptivity. The regulation of the secretion of both of these ovarian steroid hormones is, of course, under the control of the anterior pituitary and thereby ultimately regulated by the hypothalamus.

The process of nidation is dependent on the proper synchronization of ovulation and sensitivity of the endometrium to the presence of the blastocysts (26). Furthermore, like decidualization, the development and maintenance of uterine sensitivity to implanation is dependent upon the proper balance of ovarian steroids (13), the secretion of which is, at least partially, regulated by lutenizing hormone (LH) and prolactin from the anterior pituitary (1). Recently, studies by Blake, et al. (6-8) have established that nicotine may alter the timing of the ovulatory surge of LH from the anterior pituitary via action on the hypothalamus. Although the study was confined to the effects of nicotine on the timing of the critical period for LH release in ovulation, the findings raise the possibility that nicotine may similarly alter LH release not only prior to ovulation, but perhaps also during early pregnancy. Similarly, nicotine administration inhibits the proestrous surge of prolactin (9). Thus, it is possible that appropriately timed administration of nicotine may alter LH and/or prolactin release in such a

Therefore, whereas progestogens render the endometrium manner as to impair the delicate balance of ovarian steroids required to maintain uterine receptivity to the blastocyst, and thereby delay and/or impair uterine sensitivity to implantation.

In addition to the possibility that nicotine may alter the course of nidation via its action on the CNS it may also influence implantation via local uterine effects. For example, it has been suggested that the site of blastocyst invasion is influenced by the abundance of intrauterine vasculature, implantation occurring preferentially near an area of increased intrauterine pO_2 (39). Since nicotine is known to be a vaso-active substance, it is possible that the alkaloid may influence blastocyst attachment by altering the endometrial vasculature, thereby modifying oxygen availability.

That nicotine may exert an influence in implantation via action of the blastocyst itself as well as on the endometrium is suggested by the study of Fabro and Sieber (16). Following iv injection of G-(^{3}H)-nicotine into rabbits, radioactivity was detected in the endometrium, uterine fluid, and blastocyst. The data suggest that nicotine may be taken up prior to implantation by the blastocyst.

3. Experimental Designs

A. Treatments

Hypotheses I & II, that nicotine reduces uterine sensitivity to implantation and/or delays the onset of such sensitivity will be tested in the following ways:

1) Comparing the time of onset, duration and magnitude of uterine sensitivity to decidualization in control vs. nicotine treated rats.

2) Estimating of the time and frequency of implantation in control vs. nicotine treated rats by, a) direct visual detection of early implant sites, and b) determination of the time during which free blastocysts can be recovered from the uterus by flushing.

Hypotheses III, that the delay in parturition observed in nicotine treated rats reflects the delay in the time of implantation of the blastocyst, will be tested by accurately measuring the time of parturition in control vs. nicotine treated rats.

The morning on which spermatozoa are found in the vaginal lavage will be designated Day 0 of pregnancy. Insemination will arbitrarly be assumed to have occurred the previous midnight. For each experiment, inseminated rats will be assigned randomly to one of two groups: nicotine treated and control. Rats in the treated group will be assigned randomly to one of 3 dosages of nicotine: 2, 6 and 10 mg/kg of nicotine per day. The alkaloid will be injected sc twice daily in 2 equal doses, the first administered between 8:00-9:00 am, the second between 5:00-6:00 pm. The 2 mg/kg dose, while pharmacological in the rat, is comparable to that received by a heavy smoker according to the studies of Wolff et al., (36).

In addition to studying the effects of different dosages of nicotine on the parameters selected, the effects of duration of treatment will be studied. In one series of experiments, the various dosages of nicotine will be administered only during the initial 8 days of pregnancy or pseudopregnancy. In another series, the nicotine treatment will be continued throughout the duration of pregnancy. In addition, other dose levels and schedules may be used, as suggested by the experimental results obtained.

B. Reagents

The nicotine to be used will be of the purest grade (Eastman Kodak) and prepared as a 2% stock solution in 0.85% NaCl solution for dilution as necessary to prepare the proper dosage. The stock solution will be prepared at 2-3 day intervals, stored in amber glass bottles and refrigerated.

Control rats will receive equivalent volumes of 0.85% NaCl solution via sc injections administered on the same schedule as the nicotine treated rats. Otherwise, treated and control animals will be cared for in identical fashion.

4. Materials and Methods

A. Animals, Environment and Routine Procedures

Mature virgin female rats of the Holtzman strain, 200-300 g body weight, will be maintained under controlled environmental conditions with respect to room temperature

(20 - 25°C), humidity (40-60%) and photoperiod (14 hr. light, 10 hr. darkness). Illumination will be provided by standard General Electric "cool white" fluorescent lights (color temperature 4000 - 5000 K) regulated by an automatic timer. The intensity of illumination at the levels of the cages will range from 430 to 1180 lumens/m^2. Animals will be housed 1/cage and provided free access to Purina Lab Chow and water. Vaginal smears will be recorded daily for at least 3 complete estrous cycles prior to breeding or induction of pseudopregnancy.

Pseudopregnancy will be induced by mechanical stimulation of the uterine cervix with a glass rod during proestrus and estrus. Day 0 of pseudopregnancy will be defined as the last day of vaginal cornification prior to the onset of the luteal phase; Day 1 will be the day on which the vaginal smear is predominately leukocytic.

B. Measurement of Time of Onset of Parturition

The time of onset of parturition will be measured as previously described (24). On the 19th day of pregnancy, each rat will be transferred to a special cage arrangement which permits the determination of the time of onset of parturition with no disturbance to the animal (Fig. 1). A standard wire-bottom rat cage (7" wide, 9" long & 7" high) will be provided with an aluminum floor which slopes to a 1" x 2" opening at the rear of the cage. Below the opening, chart paper will be moved at a known rate of speed by a

horizontally arranged kymograph. During parturition, light, amnionic fluid and blood will flow through the hole to drop below and stain the moving chart paper. Be measuring the position of the initial stain, the time of onset of parturition can be calculated within ± 15 minutes. Direct observations have confirmed that this procedure provides a good index of the time of onset of delivery.

Treatment of Data:

Though the apparatus for recording delivery will allow determination of parturition to within ± 15 minutes, mean times of delivery will be calculated for 6 hour intervals: all deliveries within ± 3 hours of the plotted time will be pooled to produce the plotted value. The results will be calculated as the percent of the total experimental population which delivered every 6 hours during Days 21-24 (or whatever span of time is appropriate for the experimental results) of gestation. Differences of less than 20% between comparable experiments will be arbitrarily considered to be of borderline biological significance. To determine the statistical significance of the data, the mean time of delivery will be calculated for each experiment, and analysis of variance will be performed following a confirmation of homogeneity of variance.

C. Induction of Decidualization

Deciduomata will be induced by surgical traumatization as previously described (40). In brief, each rat will be

horizontally arranged kymograph. During parturition laparotomized by a mid-line incision and one or both uterine amniotic fluid and fetus will flow through the open or cut horns will be traumatized by inserting a knife-tipped needle below and near the moving chart paper. be measured into the uterine lumen by way of a small incision placed position of the initial spike, the time of onset of stimula near the junction of the horn and the cervix. The blade will tion can be calculated within ± 15 minutes. Uterine sens be pushed into the lumen and completely to the ovarian end of various have confirmed that this procedure provides a cer the uterus. The knife edge will be pressed against the entire length of the antimesometrial surface of the uterus as it is withdrawn.

Decidualization will be induced in each rat during one of 6 times: Day 2 midnight; Day 3 noon or midnight; Day 4 noon or midnight; Day 5 noon. Five days following uterine traumitization, the rats will be weighed and sacrificed by ether asphyxiation; the uterine cornua will be removed, placed on moist bibulous paper, and trimmed of adhering fat and mesentery. The cornua will be separated and weighed on a torsion balance to the nearest 0.5 mg.

Treatment of Data:

Decidual sensitivity will be expressed in 2 ways: as mg/cornu, and as percent of an arbitrary average response to trauma during the period of maximal sensitivity in normal rats (38). Under these conditions, 100% sensitivity = 2000 mg/cornu. To simplify the interpretation of results, the "onset" of decidual sensitivity will be defined as responses between 40 and 80% of maximal. Statistical analysis of the data will be performed using the one-way analysis of variance.

5. Additional Experiments and Observations

1) Changes in timing and frequency of implantation

A. Recovery of Blastocysts

As an additional test of the ability of nicotine to alter the time of implantation, the rate of recovery of blastocysts from the uterus during the preimplantation period by saline flushings will be determined. Normally, under the conditions of the present experiments, by early Day 5 nidation has proceeded to a point that blastocysts can no longer be flushed from the uterus. Therefore, a delay in implantation will be suggested if unattached blastocysts are obtainable from nicotine treated rats at significantly later times than in control rats.

An another indication of whether or not nicotine administration results in delayed nidation, the mesometrial-anti-mesoemtrial width of the implantation sites will be measured on Day 8 of treated and control rats. Such measurements will provide an approximate indication of when implantation occurred (30).

B. Early visual Detection of Implantation

The benzyl-benzoate clearing technique described by Orsini (27) will be used to render implantation sites visible. After fixing, dehydrating, bleaching and clearing, rat uteri illuminated by oblique light reveal nidation sites as early as 5 days and 9 hours following insemination (28). A delay

in implantation will be indicated if nidation sites are not visible in nicotine treated rats until significantly later times than in control rats.

2) Changes in Birth Weight and Growth, Placenta Weight, the Frequency of Resorption, and Maternal Food Intake.

A. Birth Weight

To determine whether or not reduction of fetal mass occurs, and if so, by what mechanism(s) it takes place, a variety of experiments and observations will be performed. Following delivery, each pup will be weighed, sexed, measured for length and examined for the degree of development and presence of congenital defects. The total litter and mean neonate weights will be computed and percentage of live births noted.

B. Development

Neonates will be relaxed with ether anesthesia and the naso-anal length measured to the nearest mm with vernier calipers. In addition, the stages of development of each newborn will be assessed using the method of Christie (12). This method requires the careful examination of each newborn for specific morphological characteristics, the presence or absence of which permits an estimation of the stage of development with an accuracy of $\pm$ 1/2 day.

C. Growth

As an additional indicator of growth, rate of fetal bone growth will be assessed using procion dyes. The procion dyes

to be used are derivatives of mono-chlorotriazinyl, are water soluble, and react irreversibly with osseous tissue. Since such dyes do not dissolve during the histological preparation of stained bone, they act as indicators of bone growth (26, 31).

Although the details of timing, dosage, etc., remain to be determined, in general, the method will consist of an initial iv injection of Procion Brilliant Red II-8BS, followed at an appropriate interval by an injection of Procion Brilliant Purple H-3Bs. Shortly after birth, and following measurement of other parameters, the femurs of each neonate will be dissected free, cleaned, weighed, measured for length, and fixed for histological examination. Following decalcification and sectioning, the distance between the red and purple dye lines (resulting from the administration of Procion Brilliant Red and Purple, respectively), will be measured with an ocular micrometer. The rate of femur growth during the interval between dye injections will be computed. Three measurements will be made on each femur and the mean distance between dye lines determined for each. In an effort to eliminate biased measurements, the identity of each specimen will be coded so as to prevent the observer from knowing the treatment administered. All indices of growth, ie., naso-anal length, body weight, femur length and weight, as well as rate of femur growth, will be subjected to appropriate statistical analysis to determine if there are differences between the experimental and control groups.

D. Placental Growth

The effects of nicotine on placental growth will be determined and morphologic evidence of altered placental function will be sought. In randomly selected animals, (d. 31), direct visual observations will be made to detect the onset of labor. Following delivery of the first pup, the mother will be anesthetized and the remainder of the litter delivered by Caesarean section in order to obtain intact placentae. Such placentae will be cleaned, blotted dry, and weighed. Placental diameter will be measured with vernier calipers, prior to fixation for histological examination.

E. Frequency of Resorption

To determine if nicotine increases the frequency of fetal resorption, each mother rat will be sacrificed by ether anesthesia and the uteri removed, cleaned and weighed. The number and distribution of implantation sites will be determined. The frequency of resorption will be computed for each litter by comparing the number of young in the litter vs. the number of implantation sites.

F. Changes in Maternal Food Intake.

In order to determine if nicotine administration results in reduced maternal food intake, food will be provided in spill proof containers and daily food and water consumption will be measured. Should nicotine treatment be associated with a significant reduction in food consumption, paired feeding

D. Placental Growth

experiments will be initiated to determine what effect,

in any, and equivalent reduction of food intake exerts on

fetal growth and the time of parturition in control rats.

Controls will be paired randomly with nicotine treated

rats inseminated on the same evening. Food intake of nicotine

treated animals will be measured daily, and the paired control

rat provided with only an equivalent quantity of food.

Paired animals will be observed for time of parturition;

litters and post-parturient mothers will be subjected

to the standard series of measurements to determine if the

effects of nicotine are possibly brought about by reduced

caloric intake.

determined. The frequency of resorption will be computed for

experiments will be initiated to determine what effect,
if any, and equivalent reduction of food intake exerts on
fetal growth and the time of parturition in control rats.
Controls will be paired randomly with nicotine treated
rats terminated on the [illegible] day. Food intake of nicotine
treated animals will [illegible] and the ratio control
rat provided only [illegible] food.
Maternal arterial [illegible] for time of parturition.
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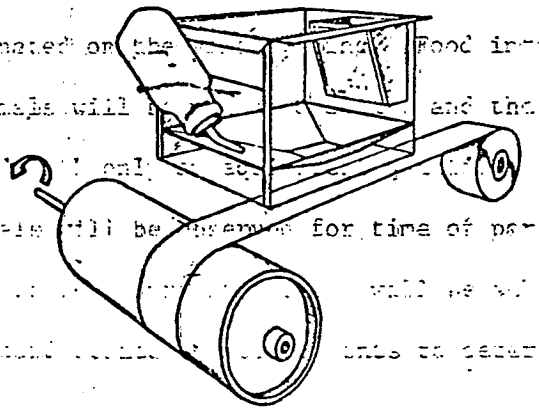

FIG. 1. Apparatus used to determine time of onset of parturition in the rat.

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